The average conformation at micromolar [Ca\(^{2+}\)] of Ca\(^{2+}\)-ATPase with bound nucleotide **differs** from that adopted with the transition state analog ADP.AlF\(_x\) or with AMPPCP under crystallization conditions at millimolar [Ca\(^{2+}\)].

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Running title : AMPPCP versus ADP.AlF\(_x\), and Ca\(^{2+}\) ion occlusion in SERCA1a

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Crystalline forms of detergent-solubilized sarcoplasmic reticulum Ca\(^{2+}\)-ATPase, obtained in the presence of either a substrate analog, AMPPCP, or a transition state complex, ADP.fluoroaluminate, were recently described to share the same general architecture despite the fact that, when studied in a test tube, these forms show different functional properties. Here, we show that the differences in the properties of the E1.AMPPCP and the E1.ADP.AlF\(_x\) membraneous (or solubilized) forms are much less pronounced when these properties are examined in the presence of 10 mM Ca\(^{2+}\) (the concentration prevailing in the crystallisation media) than when they are examined in the presence of the few µM Ca\(^{2+}\) known to be sufficient to saturate the transport sites. This concerns various properties including ATPase susceptibility to proteolytic cleavage by Proteinase K, ATPase reactivity towards SH-directed Ellman's reagent, ATPase intrinsic fluorescence properties (here described for the E1.ADP.AlF\(_x\) complex for the first time), and also the rates of \(^{45}\)Ca\(^{2+}\)-\(^{40}\)Ca\(^{2+}\) exchange at site “II”. These results solve the above paradox at least partially, and suggest that the presence of a previously unrecognized Ca\(^{2+}\) ion in the Ca\(^{2+}\)-ATPase AMPPCP crystals should be re-investigated. **A contrario**, they emphasize the fact that the average conformation of the E1.AMPPCP complex under usual conditions in the test tube **differs** from that found in the crystalline form. The extended conformation of nucleotide revealed by the E1.AMPPCP crystalline form might be only indicative of the requirements for further processing of the complex, towards the transition state leading to phosphorylation and Ca\(^{2+}\) occlusion.

After the initial description of the high resolution structures of two crystalline forms of the sarcoplasmic reticulum calcium pump (the membranous Ca\(^{2+}\)-dependent P-type ATPase SERCA1a) (1,2), additional forms of this Ca\(^{2+}\)-ATPase were recently crystallized, with the hope of characterizing as many as possible of the different intermediates formed in sequence during the catalytic cycle of this enzyme and therefore to provide a structural basis for the mechanistic description of ion pumping (3-7). Among these forms, one has its two transport sites occupied by Ca\(^{2+}\), and its nucleotide binding site occupied by a non-hydrolyzable analog of ATP, AMPPCP; it is referred to as "E1.AMPPCP". Another one, referred to as "E1.AlFx.ADP", also has its two transport sites occupied by Ca\(^{2+}\), and it has been obtained by crystallization of the quasi-irreversible complex formed by ATPase with ADP and aluminium fluoride, a complex thought to be a fair analog of the transient species formed in the catalytic cycle immediately before the ADP-sensitive "E1P" phosphoenzyme. The overall polypeptide chain architecture was found to be very similar in these two crystalline forms (except for specific features at the catalytic site): in particular, in both forms (3-6), the M1-M2 transmembrane helices appear to be pulled up towards the cytosol and the top portion (M1') of the M1 helix gets kinked, thereby locking the conformation of Glu\(^{309}\), a residue thought to cap Ca\(^{2+}\) at one of its binding sites, “site II”, and therefore to be critical for dissociation of the two bound Ca\(^{2+}\) ions out of their binding pocket (e.g. 8-9). This locking by M1-M1’ of the conformation of Glu\(^{309}\) was considered to be responsible for the long known “occlusion” of Ca\(^{2+}\) (10) that occurs during normal turnover, after ATPase phosphorylation from ATP. In the
E1.AlFx.ADP form, it is indeed accepted that the Ca\(^{2+}\) transport sites are occluded (4, 9, 11), as in the (transient) E1P phosphoenzyme form.

However, it has been suggested previously that a similar occlusion occurs neither after the mere formation of a non-covalent E1.AMP-PCP complex, nor after the mere formation of the E1.Mg.ATP complex that immediately precedes phosphorylation during the normal cycle (4, 9, 12, 13). This apparent discrepancy between the different properties of the two ATPase forms and their similar crystalline structure has already been noted (4, 5, 9), and various interpretations have been given, together with somewhat contradictory comments that these two forms, E1.AMPPCP and E1.AlFx.ADP, have (5) or do not have (4) a similar pattern of resistance to cleavage by proteinase K of their cytosolic domains. Structural fluctuation of the non-crystallized ATPase.AMPPCP complex (we will discuss it) and/or selection of a particular conformation by crystal packing were suggested to explain the different occlusion properties of the two ATPase forms in a test tube (4, 5).

In view of the significance of this issue with respect to the mechanism of occlusion, we decided to further document the resemblance or differences between E1.AMPPCP and E1.AlFx.ADP forms. In particular, we asked whether any clues could be provided by the fact that the published crystalline forms of E1.AMPPCP had been obtained at very high Ca\(^{2+}\) concentrations. Under high millimolar Ca\(^{2+}\) conditions, we found that the ATPase complex with AMPPCP indeed has properties closer to those of the ATPase complex with ADP.fluoroaluminate than under micromolar Ca\(^{2+}\) conditions. Under high millimolar Ca\(^{2+}\) conditions, we found that the ATPase complex with AMPPCP had been obtained at very high Ca\(^{2+}\) concentrations. Under high millimolar Ca\(^{2+}\) conditions, we found that the ATPase complex with AMPPCP indeed has properties closer to those of the ATPase complex with ADP.fluoroaluminate than under micromolar Ca\(^{2+}\) conditions. Under high millimolar Ca\(^{2+}\) conditions, we found that the ATPase complex with AMPPCP indeed has properties closer to those of the ATPase complex with ADP.fluoroaluminate than under micromolar Ca\(^{2+}\) conditions.

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AMPPCP is initially only present together with quin2 (in the latter case, the Ca.AMPPCP complex is not formed); (iii) we directly checked, in the absence of membranes, that any Ca.AMPPCP or Ca.ATP complex formed under our conditions dissociates faster than the dead time (3 ms) of our stopped-flow measurements.

All these experiments were performed at 20 °C. Most of them (unless otherwise noted) were performed in a 50 mM Mes-Tris buffer at pH 6, generally without potassium, or in a 50 mM Mops-Tris buffer at pH 7, supplemented with 100 mM KCl and 5 mM Mg\(^{2+}\). AMPPCP and ATP\(\gamma\)S were from Sigma, ADP was from Fluka, and quin2 was from Calbiochem. Equilibrium fluorescence experiments were performed with a SPEX Fluorolog fluorometer, with constant stirring of the temperature-controlled cell. Stopped-flow experiments were performed with Bio-Logic SFM3 equipment; quin2-emitted fluorescence was detected with a broad band filter, DA 531 from MTO (Massy, France).

The extent of Ca\(^{2+}\)-ATPase proteolysis by proteinase K under different conditions was examined by SDS-PAGE in 12 % acrylamide gels prepared in the presence of 1 mM Ca\(^{2+}\), as described in ref. 18. SH reaction with DTNB (Ellman's reagent, from Sigma) was monitored at 25°C, using a diode array spectrophotometer (HP 8453) operated in the kinetic mode. DTNB conversion to a colored anion upon reaction with SH groups was monitored at 430 nm, using an extinction coefficient of 12 mM\(^{-1}\)cm\(^{-1}\), slightly lower than that at the wavelength for maximal absorption (13.6 mM\(^{-1}\)cm\(^{-1}\) at 412 nm); 430 nm, instead of the more widely used wavelength of 412 nm, was chosen to minimize the slight interference with DTNB absorption which occurs at 412 nm. Absorption by DTNB is not visible in Figure 4 because DTNB was already present in the buffer used to record the blank spectrum.

Note that judging from the apparent affinities reported in ref. 19 for AMPPCP-cation complexes, the dissociation constants of AMPPCP complexes with Mg\(^{2+}\) and Ca\(^{2+}\) in 50 mM Mes-Tris at pH 6 probably are in the 0.4-0.5 mM and the 0.8-1 mM ranges, respectively (i.e. intermediate between those for ATP and those for ADP, as also shown by our own measurements with antipyrylazo III, data not shown).

### RESULTS

**ADP.AlF\(_4\) blocks \(^{45}\)Ca\(^{2+}\) dissociation from SR Ca\(^{2+}\)-ATPase, but under ordinary conditions, AMPPCP does not.**

Sørensen et al. (4) and Toyoshima and Mizutani (5) found that the presence of AMPPCP or ADP.fluoroaluminate in the crystalline forms of Ca\(^{2+}\)-ATPase resulted in structural changes indicative of occlusion of the bound Ca\(^{2+}\) ions in both cases, but they already noted that this was somewhat dissonant with a previous experimental finding deduced from rapid filtration experiments with \(^{45}\)Ca\(^{2+}\): the half time for the dissociation of \(^{45}\)Ca\(^{2+}\) from the transport sites of non-phosphorylated ATPase only increases by 50 % in the presence of 250 µM AMPPCP, at pH 6 in the presence of 20 mM Mg\(^{2+}\) (12), and thus dissociation remains relatively fast in the presence of AMPPCP. We first repeated that old experiment under different conditions, and at pH 7 in the presence of 5 mM Mg\(^{2+}\), found a similar although not identical result: AMPPCP again failed to block \(^{45}\)Ca\(^{2+}\) dissociation, but in these experiments did not even slow down the rate of this dissociation at all, even if its concentration was increased up to 2.5 mM. Yet, under the same conditions, preliminary incubation of Ca\(^{2+}\)-ATPase with ADP and fluoroaluminate did block \(^{45}\)Ca\(^{2+}\) dissociation completely (see Figure I in Supplemental Material, and accompanying comments).

Depending on Mg\(^{2+}\), AMPPCP may either stimulate or slightly reduce the rate of overall Ca\(^{2+}\) dissociation from Ca\(^{2+}\)-ATPase, and the modulatory effect of Mg\(^{2+}\) differs for various nucleotides.

We therefore reinvestigated the effect of AMPPCP under the previous conditions at pH 6, using a stopped-flow assay in which Ca\(^{2+}\) dissociation from Ca\(^{2+}\)-ATPase was triggered by mixing Ca\(^{2+}\)-equilibrated SR vesicles with the fluorescent chelator quin2, whose fluorescence changes allow to monitor the rate of this Ca\(^{2+}\) dissociation (see Materials and Methods, and Figure II in Supplemental Material). With this new assay, we found that at pH 6 too, AMPPCP in the presence of a few mM Mg\(^{2+}\) only minimally affected Ca\(^{2+}\) dissociation from ATPase (Panel A of Figure 1), but we nevertheless confirmed that in the presence of 20 mM Mg\(^{2+}\) AMPPCP did slow down Ca\(^{2+}\) dissociation moderately, as previously observed in \(^{45}\)Ca\(^{2+}\) filtration experiments (12); at lower
Mg$^{2+}$ concentrations, however, this slowing down was in fact converted into a definite acceleration of the rate of Ca$^{2+}$ dissociation, by a factor of up to 4 in the total absence of Mg$^{2+}$ (representative traces are shown in Figure III of Supplemental Material). This is summarized in Panel B of Figure 1, which includes experiments performed at high AMPPCP concentrations in the presence of 20 mM Mg$^{2+}$. These data reveal no real occlusion of Ca$^{2+}$ after the mere binding of AMPPCP.

In the absence of Mg$^{2+}$, the EC$_{50}$ for AMPPCP-induced acceleration of Ca$^{2+}$ dissociation was in the micromolar range (open circles in Panel B; the true EC$_{50}$ is somewhat lower than the one apparent in this Panel, because of sub-stoichiometric conditions for the lowest concentration of AMPPCP tested). Incidentally, note that although the quin2 fluorescence rise that we measured monitors the overall dissociation of the two Ca$^{2+}$ ions previously bound to Ca$^{2+}$-ATPase (see Experimental Procedures and Figure II in Supplemental Material), all traces were quasi-monophosphoric, as previously noted and interpreted within the context of a "flickering gate" for the Ca$^{2+}$-binding pocket (20, 12, 17).

AMPPCP is not a perfect analog of ATP, as the Mg$^{2+}$-dependence of its binding to Ca$^{2+}$-ATPase is opposite to the Mg$^{2+}$-dependence of the binding of ATP itself (19, 21, 22; this will be confirmed below), presumably because binding in the presence of Mg$^{2+}$ is perturbed by the CH$_2$ link between $\beta$ and $\gamma$ phosphates in AMPPCP. Therefore, for comparison, we also measured the rate of Ca$^{2+}$ dissociation from ATPase in the presence of another poorly-hydrolyzable analog of ATP, ATP$_{\gamma}$S (adenosine 5'-[\$\gamma\$-thio]triphosphate), whose Mg$^{2+}$-dependence has been shown to retain an ATP-like behaviour, i.e. a higher affinity in the presence of Mg$^{2+}$ than in its absence (23). ATP$_{\gamma}$S was found to stimulate the rate of Ca$^{2+}$ dissociation both in the absence and presence of Mg$^{2+}$, and in this case Mg$^{2+}$ enhanced the ATP$_{\gamma}$S-dependent acceleration instead of converting it into a slowing down (original traces in Figure IV of Supplemental Material). Note that for these experiments, ATP$_{\gamma}$S (at twice the final concentration) was purposely added together with quin2, and thus, SR vesicles were not preincubated with ATP$_{\gamma}$S: this is because ATP$_{\gamma}$S, although poorly hydrolysed (24), has been shown to react with Ca$^{2+}$-ATPase in the presence of Ca$^{2+}$, leading to accumulation of a thio-phosphorylated intermediate (25). This alteration in our experimental protocol (compared with the above-described case of AMPPCP) was tested in the case of AMPPCP, and it had no significant influence on the rate constant for Ca$^{2+}$ dissociation; note that nucleotide-dependent rates of Ca$^{2+}$ binding have previously also been found similar when the nucleotide is added simultaneously with Ca$^{2+}$ and when nucleotide is preincubated with Ca$^{2+}$-free ATPase (26), presumably because of the fairly fast rate of nucleotide binding at a concentration of 250 µM. Because of the puzzling difference between AMPPCP and ATP$_{\gamma}$S in their Mg$^{2+}$ dependencies, we performed a third series of experiments with ADP (again added together with quin2) in the presence of various concentrations of Mg$^{2+}$: 1 mM ADP accelerated Ca$^{2+}$ dissociation at all concentrations of Mg$^{2+}$. Panel C in Figure 1 summarizes all these results.

Note that experiments were also performed at pH 6 in the presence of 100 mM KCl and 5 mM Mg$^{2+}$. In this series of experiments, 250 µM AMPPCP again hardly affected the rate of Ca$^{2+}$ dissociation (from 14 to 15 s$^{-1}$), while the same concentration of ADP accelerated it moderately (to 23 s$^{-1}$) (data not shown). In previous experiments performed at pH 6.5 in the presence of 100 mM KCl and 2 mM Mg$^{2+}$ at 11 °C, ADP had also been found to only have a weak effect on Ca$^{2+}$ dissociation (15). This confirms that although the presence of potassium slightly accelerates Ca$^{2+}$ dissociation (e.g. 12, 17), it does not affect the above-described pattern of nucleotide-induced alteration: the presence of nucleotide generally does not lead to Ca$^{2+}$ occlusion.

If free Ca$^{2+}$ is increased to high millimolar concentrations, well beyond those allowing saturation of the ATPase high affinity sites, AMPPCP binds to Ca$^{2+}$-ATPase with even higher affinity.

As a preliminary step in our attempt to understand the effect of AMPPCP on Ca$^{2+}$-ATPase under crystallization conditions, we estimated the affinity with which AMPPCP binds to Ca$^{2+}$-ATPase under such conditions, namely in the presence of a high millimolar Ca$^{2+}$ concentration. For this purpose, we used a Trp fluorescence assay (27), and first checked that it gave results consistent with the above-mentioned effect of Mg$^{2+}$ on AMPPCP binding (19, 21, 22).
That this was the case, both in the absence and in the presence of an ordinary submillimolar concentration of Ca\(^{2+}\), is shown in Panels A-D of Figure 2 (for control, the well known opposite dependence on Mg\(^{2+}\) of ATP binding in the absence of Ca\(^{2+}\) is shown in Figure V of Supplemental Material); note that the equilibrium dissociation constant for AMPPCP binding deduced from the assay in the presence of Ca\(^{2+}\) and absence of Mg\(^{2+}\) (Figure 2C) is slightly lower than the apparent EC\(_{50}\) with which AMPPCP accelerates the rate of Ca\(^{2+}\) dissociation from its binding sites in the absence of Mg\(^{2+}\) (previously illustrated in Figure 1B): this is probably in part due to the fact that nucleotide binding at a concentration of only a few µM may be rate limiting.

We then monitored the binding of various concentrations of AMPPCP in the presence of a high, millimolar Ca\(^{2+}\) concentration, and found that the affinity for AMPPCP binding was increased further, compared to its affinity in the presence of submillimolar Ca\(^{2+}\) concentrations sufficient to saturate the Ca\(^{2+}\) transport sites: this was found both in the presence of Mg\(^{2+}\) (Figure 2, E-F) and in its absence (Figure VI of Supplemental Material). Related results are scattered in the literature (19, 21, 22). A high millimolar Ca\(^{2+}\) therefore affects AMPPCP binding in some way.

**AMPPCP binding at millimolar Ca\(^{2+}\) (but not submillimolar Ca\(^{2+}\)) almost fully protects ATPase from proteolysis by proteinase K.**

We first estimated the effect of a high millimolar Ca\(^{2+}\) on AMPSPCP-dependent changes through measurements of the susceptibility of Ca\(^{2+}\)-ATPase to proteolysis. The simultaneous presence of AMPPCP and Ca\(^{2+}\) (18, 28, 29) was previously described as affording partial protection of Ca\(^{2+}\)-ATPase from digestion by proteinase K, and the combination of fluoroaluminate with ADP in the presence of Ca\(^{2+}\) was also described previously to afford protection (28, 9). In one of the analyses of the X-ray data, the increased resistance of both the E1.AMPPCP and E1.AlFx.ADP forms against proteolysis was considered to be consistent with the similar structures of the two crystalline forms (5). In contrast, in another analysis (4), the protection afforded by AMPPCP was reported to be only minimal compared to the strong protection afforded by fluoroaluminate in the presence of ADP and Ca\(^{2+}\), and this was considered instead to be consistent with the lack of Ca\(^{2+}\) occlusion by E1.AMPPCP, despite the similar structures (4). In the present work, we have tried to solve these apparent discrepancies by giving particular attention to the Ca\(^{2+}\) concentration prevailing during proteolysis.

We have found that extensive protection by AMPPCP is obtained in the presence of millimolar Ca\(^{2+}\) concentrations similar to those used for crystallization. This is shown in Figure 3, a quasi-repetition of Figure S2 in Sørensen et al. (2004), except that in addition to monitoring ATPase proteolysis in the presence or absence of AMPPCP, in the presence of 0.1 mM Ca\(^{2+}\) (and the presence of 1 mM Mg\(^{2+}\), at pH 6.5) (Panel A), we repeated the experiment in the presence of 10 mM Ca\(^{2+}\) (Panel B): the combination of AMPPCP and a high Ca\(^{2+}\) concentration provided almost full protection against proteolysis, in contrast with the only poor protection observed at 0.1 mM Ca\(^{2+}\). Similar results were also obtained at pH 7.2, as in Sørensen et al.’s experiments (see Figure VII in Supplemental Material). Thus, at high Ca\(^{2+}\), AMPPCP does exert effects similar to those exerted by ADP.fluoroaluminate.

A millimolar Ca\(^{2+}\) concentration also makes AMPPCP more efficient for protecting the ATPase sulfhydryl groups from modification by DTNB.

Measuring the susceptibility of ATPase cysteines to modification by DTNB (Ellman’s reagent) was reported to be another way of differentiating the E1.AMPPCP and E1.AlFx.ADP forms, as the susceptibility of the former was almost unaltered compared to control, whereas that of the latter was drastically reduced (4). We repeated this experiment, using alkaline conditions classically used to deprotonate sulfhydryl groups and therefore render them reactive to DTNB. Reaction of SH groups with DTNB could be reliably deduced from the development of an absorption band characteristic of the appearance of 2-nitro-5-thiobenzoate (e.g. Panels A & B in Figure 4), with essentially no complication arising from turbidity problems (as checked at higher wavelengths, e.g. 550 nm, see Panel D). As in ref. 4, we found that one or two SH group(s) per ATPase remained very reactive to DTNB; reaction of SH groups with DTNB could be reliably deduced from the development of an absorption band characteristic of the appearance of 2-nitro-5-thiobenzoate (e.g. Panels A & B in Figure 4), with essentially no complication arising from turbidity problems (as checked at higher wavelengths, e.g. 550 nm, see Panel D).
of AMPPCP with a submillimolar Ca\(^{2+}\) concentration (Panel C in Figure 4). The combination of AMPPCP and a high millimolar Ca\(^{2+}\) reduced SH susceptibility (although again not to zero) also at pH 7.5 (Figure VIII in Supplemental Material) as well as at pH 7.2 (data not shown), instead of pH 8. In those cases, however, aggregation soon followed the reaction of SH groups with DTNB in the presence of 10 mM Ca\(^{2+}\), so that the picture was less nice (at least when SR vesicles were used, not purified ATPase as in ref. 4); however, in that case, the presence of AMPPCP delayed aggregation very significantly, which provides additional evidence that AMPPCP in the presence of 10 mM Ca\(^{2+}\) exerted significant protection on SH groups critical for aggregation (Panels B & D in Figure VIII). SH groups reactivity was also reduced, although not again to zero, in the presence of ADP/fluoroaluminate (Figure IX in Supplemental Material).

AMPPCP slows down 45Ca\(^{2+}\)-40Ca\(^{2+}\) exchange considerably at very high [40Ca\(^{2+}\)], to a larger extent than a similar concentration of Mg\(^{2+}\).

Thus, Figures 3 and 4 suggest that AMPPCP in the presence of a high millimolar Ca\(^{2+}\) concentration exerts effects on Ca\(^{2+}\)-ATPase that are different from (or more pronounced than) those exerted at micromolar Ca\(^{2+}\) concentrations. To study whether the simultaneous presence of AMPPCP and a very high Ca\(^{2+}\) concentration results in significant slowing of the flickering movements of the gate capping the more "superficial" Ca\(^{2+}\) binding site -occlusion implies complete freezing of these movements-, we resorted to 45Ca\(^{2+}\) filtration experiments, specifically to 45Ca\(^{2+}\)-40Ca\(^{2+}\) exchange experiments, in which 45Ca\(^{2+}\) ions initially bound to the transport sites are allowed to exchange with 40Ca\(^{2+}\) in the perfusion medium; under these conditions, it is known that only one 45Ca\(^{2+}\) ion, the one bound to the more accessible site (site "II", gated by E309), is freely exchangeable, while dissociation of the Ca\(^{2+}\) ion bound to the second site (site "I") is greatly retarded by the 40Ca\(^{2+}\) ion just bound (e.g. 12, 30).

As a starting point, we knew that in the absence of AMPPCP the concentration of 40Ca\(^{2+}\) in the perfusion medium could be raised up to 30 mM without affecting the rate of this 45Ca\(^{2+}\)-40Ca\(^{2+}\) exchange (e.g. Figure 6 in ref. 12). We also knew that at pH 6 in the presence of 20 mM Mg\(^{2+}\), 45Ca\(^{2+}\)-40Ca\(^{2+}\) exchange at 1 mM 40Ca\(^{2+}\) was only slowed down by AMPPCP to a moderate extent, similar to that for overall 45Ca\(^{2+}\) dissociation in the presence of EGTA (Table I in ref. 12). The critical test, thus, was to perform a similar experiment with AMPPCP together with a still higher 40Ca\(^{2+}\) concentration. This was first done in the presence of 3 mM Mg\(^{2+}\): Figure 5A shows that in the presence of 10 mM 40Ca\(^{2+}\) (a concentration which per se did not slow down 45Ca\(^{2+}\)-40Ca\(^{2+}\) exchange), but not in the presence of only 1 mM 40Ca\(^{2+}\), the additional presence of AMPPCP in the perfusion medium slowed down exchange considerably, i.e. the previously bound 45Ca\(^{2+}\) ions were now "quasi-occluded" in the presence of AMPPCP. This was also the case in the presence of 20 mM 40Ca\(^{2+}\) (see Figure X in Supplemental Material).

In the absence of Mg\(^{2+}\), where AMPPCP in the absence of 40Ca\(^{2+}\) stimulated 45Ca\(^{2+}\) dissociation (as also shown in Figure 1B above), AMPPCP in the presence of 10 mM 40Ca\(^{2+}\) again slowed down 45Ca\(^{2+}\)-40Ca\(^{2+}\) exchange, and 10 mM 40Ca\(^{2+}\) was more effective than 10 mM Mg\(^{2+}\) together with 1 mM 40Ca\(^{2+}\) (Figure 5B). Thus, AMPPCP in the presence of the high Ca\(^{2+}\) concentrations used to obtain the crystalline form of E1.AMPPCP induced significant slowing down of the flickering movements of the gate capping the more superficial Ca\(^{2+}\) binding site, a slowing down resembling what ADP/fluoroaluminate also does to fully occlude Ca\(^{2+}\) ions. It is however fair to recognize that even at high 40Ca\(^{2+}\) concentrations, 45Ca\(^{2+}\) retention in the presence of AMPPCP did not persist for very long periods (e.g. minutes), in contrast with 45Ca\(^{2+}\) occlusion in the E1.ADP.AlFx complex (4).

In the presence of 50 µM Ca\(^{2+}\), ADP/AlF\(_4^-\) raises the ATPase intrinsic fluorescence to a level higher than that of E1.AMPPCP (in contrast with AMPPCP, this involves Trp residues sensitive to quenching by A23187), but in the presence of 10 mM Ca\(^{2+}\) the difference is less prominent.

We also compared the intrinsic Trp fluorescence of the E1.ALFx.ADP and E1.AMPPCP forms. To start with, we reproduced Troullier et al.'s Trp fluorescence observations with SR Ca\(^{2+}\)-ATPase incubated with fluoroaluminate in the absence of Ca\(^{2+}\) (Fig 1 of ref. 11): under our experimental conditions (100 mM KCl, 50 mM Mops-Tris, 5 mM Mg\(^{2+}\), pH 7, 20°C, with 1 mM KF and 50 µM AlCl\(_3\), here in the presence of 240 µM
EGTA), ATPase inhibition by fluoroaluminate in the absence of Ca\(^{2+}\) was only accompanied by an initial hardly resolvable increase in fluorescence followed by slow reduction of fluorescence (as in Troullier's paper) (Figure XI in Supplemental Material).

In contrast, when the ATPase reaction with fluoroaluminate proceeded in the presence of 50 \(\mu\text{M}\) free Ca\(^{2+}\) and 100 \(\mu\text{M}\) ADP, instead of EGTA alone, the Trp fluorescence of Ca\(^{2+}\)-ATPase now rose significantly (Panel A in Figure 6). The amplitude of this rise was of a few per cent, like most other changes for Ca\(^{2+}\)-ATPase, and its time course was consistent with the fast inhibition that we measured in parallel in ordinary assays of the residual ATPase activity (data not shown). KF alone had no effect. After reaction with ADP/fluoroaluminate, the Trp fluorescence level was no longer sensitive to addition of excess EGTA (2 mM; double arrow in Panel A), in agreement with the previously mentioned formation of a stable ATPase complex from which occluded Ca\(^{2+}\) cannot dissociate (and Figure I of Supplemental Material). The rate of the Trp fluorescence rise after addition of AlCl\(_3\) in the presence of ADP and fluorur was dependent on the presence of Mg\(^{2+}\), with an apparent affinity of a few millimolar (data not shown), and this is probably why it was not detected in Troullier et al.'s previous experiments, performed in the presence of 1 mM Mg\(^{2+}\) only.

Thus, it appears that formation of a complex with ADP/fluoroaluminate in the presence of Ca\(^{2+}\) and Mg\(^{2+}\) brings the Ca\(^{2+}\)-saturated ATPase fluorescence to a level higher than that of the “Ca\(_2\),E1” form, and even higher than that of the “E1.ADP” form present after addition of ADP but before addition of aluminium. On the other hand, AMPPCP alone brings the Ca\(^{2+}\)-saturated ATPase up to a level only slightly higher than that produced by ADP (Panel B in Figure 6). Thus, it is clear that under submillimolar Ca\(^{2+}\) conditions, the fluorescence level of the E1.AIFx.ADP form is different from that of the E1.AMPPCP form.

Yet, Panels C and D in Figure 6 indicate that in the presence of 10 mM Ca\(^{2+}\), the difference between the fluorescence levels for these two forms is smaller: the fluorescence rise due to AMPPCP-binding is larger (compare D to B), while that due to formation of the E1.AIFx.ADP complex does not change much or is even reduced, as the ADP-induced rise is also larger but the AlCl\(_3\)-induced rise is smaller (compare C to A). Although signals are not very large, this trend is highly reproducible from one experiment to the other (see also below).

We attempted to classify the ATPase Trp residues involved in these fluorescence responses according to their sensitivity to short-range fluorescence quenchers located within the membrane, either ionophore A23187 (calcimycin), which quenches nearby Trp fluorophores through Förster resonance transfer (31), or ionophore BrA23187, which in addition to a less efficient Förster transfer mechanism probably also quenches them by a bromine-mediated quenching mechanism (e.g. 32, 33). In both cases, Trp\(^{552}\), the only Trp residue of Ca\(^{2+}\)-ATPase located in the cytosolic domain, is likely not to be quenched. When measurements similar to those shown in Panels A & B of Figure 6 were repeated in the presence of 4 \(\mu\text{g/ml}\) A23187 (such an amount reduces overall ATPase fluorescence to only 30 % of its initial fluorescence, corresponding roughly -assuming equal contributions of the various Trp residues- to the fluorescence of 4 out of the 13 Trp residues), the initial changes observed upon addition of EGTA and then Ca\(^{2+}\), plotted on the same vertical scale as previously (see Figure XII of Supplemental Material), were much smaller (compare Panels C & D in Figure XII to Panels A & B). This was previously found and interpreted as implying that these changes mainly arise from Trp residues located within or close to the membrane (and thus easily quenched by A23187) (31, 33). In contrast, the rise in fluorescence induced by either AMPPCP (Panel D) or ADP (Panel C) in the presence of Ca\(^{2+}\) was not quenched, suggesting that perhaps it mainly arose from Trp\(^{552}\) in domain N; but conversely, most of the rise in fluorescence subsequently induced by AlCl\(_3\) after addition of fluoride was quenched (Panel C in Figure XII), suggesting it mainly arose from Trp residues in the membrane domain. All this suggests that the specific rise in fluorescence accompanying formation of the E1.AIFx.ADP species in the cuvette involves Trp residues different from those involved in response to formation of the E1.AMPPCP form, presumably Trp residues within or close to the membrane domain. Judging from available structures, Trp\(^{50}\), Trp\(^{77}\) and Trp\(^{109}\) in M1 and M2 move quite significantly when going from the E1.Ca\(_2\) form to the E1.ADP.AIFx crystalline form (PDB references 1WPE versus 1SU4; see Figure 7), because of movement towards the cytosol of the
M1-M2 transmembrane hairpin. As Trp$^{50}$ and Trp$^{107}$ seem to remain close to the membrane interface in both of these forms, Trp$^{77}$, by entering the hydrophobic region of the membrane because of this movement, could be specifically responsible for the observed rise in fluorescence.

**In the presence of solubilizing detergent in addition to 10 mM Ca$^{2+}$, the difference between E1.AMPPCP and the E1.ADP.AlF$_4$ forms is even less prominent.**

We finally asked whether the effect of 10 mM Ca$^{2+}$ on E1.AMPPCP would resist SR membrane solubilization by detergent, a step involved in the crystallization procedure. This was first checked using Trp fluorescence as conformational index. Figure 8 illustrates an experiment similar to the one in Figure 6, but now performed in the presence of 2.5 mg/ml dodecylmaltoside (“DDM”, about 5 mM), a concentration in large excess over that of SR vesicles (0.1 mg/ml) and over the detergent cmc (0.18 mM) and therefore able to solubilize these vesicles completely. Qualitatively, effects of 10 mM Ca$^{2+}$ were similar to those described above, but quantitatively, the effect of 10 mM Ca$^{2+}$ on AMPPCP-induced and ADP.AlF$_4$-induced fluorescence changes was even more apparent for solubilized ATPase in Figure 8 than for native SR membranes in Figure 6: in the presence of detergent and 10 mM Ca$^{2+}$, AlCl$_3$ addition only had a modest effect on top of the increased effect of ADP (Panel C versus A), while AMPPCP now produced a major increase in fluorescence (Panel D versus B).

The same trend was observed when either C$_{12}$E$_8$ (Figure XIII in Supplemental Material) or dodecyl phosphocholine (DPC) was used (at 5 mM, data not shown) for solubilization of SR membranes, confirming that the effect of a high Ca$^{2+}$ on AMPPCP-dependent changes was more pronounced for solubilized Ca$^{2+}$-ATPase (i.e. under crystallization conditions) than for intact membranes, irrespective of the particular detergent. In the former case, similar results were obtained at three different concentrations of C$_{12}$E$_8$, 0.25, 2.5 or 10 mg/ml (i.e. about 0.5, 5 or 20 mM) (data not shown), suggesting there was no effect of ATPase delipidation. In this case, experiments were also repeated in the presence of A23187 ionophore (previously used to quench fluorescence of the Trp residues located near within or close to the transmembrane region), at the lowest (yet fully solubilizing) concentration of detergent: under these conditions the AMPPCP-dependent increase in Trp fluorescence was no longer significantly larger at 10 mM Ca$^{2+}$ than at 50 µM Ca$^{2+}$ (Figure XIV in Supplemental Material). This is again consistent with the idea that those Trp residues which are responsible for the increased fluorescence in the presence of high Ca$^{2+}$, AMPPCP and detergent, are the same as those which give rise, under ordinary conditions, to the ADP.AlF$_4$-dependent fluorescence rise.

We finally took advantage of the larger fluorescence changes observed with solubilized Ca$^{2+}$-ATPase to ask whether Mg$^{2+}$ and Ca$^{2+}$ at high concentrations had similar effects. The answer was no: an additional 20 mM Mg$^{2+}$ had a weak effect on AMPPCP- and ADP.AlF$_4$-dependent signals in a direction opposite to that of 10 mM Ca$^{2+}$, but did not prevent 10 mM Ca$^{2+}$ from exerting its own effect (Figure XV of Supplemental Material).

Beyond these Trp fluorescence experiments, we also used our SH reactivity assay to evaluate whether the above finding that solubilization made the conformation of ATPase in the presence of AMPPCP and high Ca$^{2+}$ look even more similar to that in the presence of ADP.AlF$_4$, was also true for a completely different conformational index. The answer was yes, again both for C$_{12}$E$_8$-solubilized ATPase (Figure XVI of Supplemental Material) and for DPC-solubilized ATPase (data not shown).

**DISCUSSION**

The above-described experiments confirm the idea that under usual conditions AMPPCP only has a very modest effect on the rate of Ca$^{2+}$ dissociation from non-phosphorylated Ca$^{2+}$-ATPase. At high Mg$^{2+}$ concentration, this effect consists in a slight slowing down of Ca$^{2+}$ dissociation, as previously reported (12), while at lower Mg$^{2+}$ concentration, acceleration is even observed, as now documented in Figure 1. Acceleration of the rate of Ca$^{2+}$ dissociation in fact seems to be a fairly general feature of the effect of nucleotides, as it is observed at all Mg$^{2+}$ concentrations with both ADP and ATP$γ$S, the latter nucleotide being a poorly hydrolyzable analog of ATP probably resembling ATP more than AMPPCP does (23). As concerns ATP itself, detailed time-resolved phosphorylation experiments...
previously suggested also that the rate of Ca\(^{2+}\)-dissociation from Ca\(^{2+}\)-ATPase was slightly enhanced (but not much) in the presence of Mg.ATP: Ca\(^{2+}\) dissociation from the non-covalent E.Ca\(^{2+}\).ATP complex was estimated to occur with a rate constant in the range of 45 to 80 s\(^{-1}\) at 25°C and pH 7 in the presence of 100 mM KCl and 5 mM Mg\(^{2+}\) (13), compared to 25 or 50 s\(^{-1}\) in the absence of ATP (depending on whether overall dissociation of the two Ca\(^{2+}\) ions or dissociation of the most accessible one is considered) (34). Thus, under ordinary conditions, Ca\(^{2+}\) dissociation does not appear to be blocked by the mere binding of nucleotide. In the reverse direction, Ca\(^{2+}\) binding to these transport sites was previously demonstrated to be accelerated by nucleotides, including AMPPCP (see references and results in ref. 26), a fact which again would be hard to understand if the Ca\(^{2+}\)-binding sites in E1.AMPPCP were to be occluded. Trying to reconcile the now-established fact that Ca\(^{2+}\) is strongly occluded in the ATPase.ADP.fluoroaluminate complex but not in the ATPase.AMPPCP complex (under usual conditions in a test tube) with the other now-established fact that a common architecture is found for both complexes in their crystalline forms (3-5), will be the first purpose of the present discussion.

One of our main observations in this work has been that the presence of both AMPPCP and a high millimolar Ca\(^{2+}\) concentration (instead of the micromolar Ca\(^{2+}\) concentration sufficient for saturating the ATPase high affinity transport sites) definitely affects the ATPase properties and makes the E1.AMPPCP complex look more similar to the E1.ADP.AlFx complex, from the point of view of a number of parameters (Figures 3-6), including the kinetics of \(^{45}\)Ca\(^{2+}\)–\(^{40}\)Ca\(^{2+}\) exchange (from which the initial paradox we are trying to solve is derived). This resemblance is even more significant for detergent-solubilized Ca\(^{2+}\)-ATPase (e.g. Figure 8), i.e. under the conditions prevailing during crystallization. Thus, the simplest way out of our paradox is to consider that the reason why the two crystalline forms of ATPase prepared with AMPPCP or ADP.AlFx have the same architecture (4, 5) is that under the conditions of crystallization at very high [Ca\(^{2+}\)] they are rather similar, even if this is not the case under ordinary conditions in a test tube.

One possible reason for this effect of high Ca\(^{2+}\) concentrations could be extra-binding of Ca\(^{2+}\) to a site not recognized in previous descriptions of the X-ray E1.AMPPCP structure: either at the phospholipid/water interface, or close to the negatively charged amphiphilic top portion of M1, or, more simply, at the catalytic site for phosphorylation in the cytosolic domain, previously described as containing Mg\(^{2+}\) (4, 5): in the latter view, experiments performed at high Ca\(^{2+}\) would simply reveal the properties of ATPase complexed with Ca.AMPPCP instead of AMPPCP or Mg.AMPPCP, i.e. the specific properties of an ATPase.nucleotide complex with Ca\(^{2+}\) instead of Mg\(^{2+}\) bound at the catalytic site. It is well known that at high concentrations of Ca\(^{2+}\) (or Ln\(^{3+}\)), Ca.ATP (or Ln.ATP) may substitute for Mg.ATP for binding to the catalytic site, and that the bound Ca\(^{2+}\) (or Ln\(^{3+}\)) slows down phosphoenzyme processing; remarkably, La\(^{3+}\) was previously also found, in \(^{45}\)Ca\(^{2+}\) dissociation experiments, to reduce the rate of dissociation of both \(^{45}\)Ca\(^{2+}\) ions (30, 35). In fact, Ca\(^{2+}\) itself appears to be able to bind to the corresponding catalytic site in phosphoserine phosphatase, though with an altered coordination geometry (7 instead of 6 coordinations), if the crystallization buffer contains 0.7 M Ca\(^{2+}\) (36). Thus, the identity of the metal bound to the catalytic site in the crystalline E1.AMPPCP complex of ATPase might be worth reexamining, even though coordination geometries, distances, and B factors in the E1.AMPPCP crystalline form initially suggested that this metal was mostly Mg\(^{2+}\) (4, 5). This question could be re-examined by measuring anomalous scattering by Ca\(^{2+}\) at an appropriate X-ray wavelength (Nissen, personal communication). On the other hand, if it turns out that E1.AMPPCP crystals do not contain more than contaminating amounts of bound Ca\(^{2+}\) (instead of Mg\(^{2+}\)) at the catalytic or nucleotide site, or even elsewhere (besides the transport sites), we will have to find an alternative explanation for the effect of high Ca\(^{2+}\) concentrations, consistent with the absence of detectable Ca\(^{2+}\) in the crystal. For instance, if we assume that a protein’s site for Mg\(^{2+}\) cannot accommodate Ca\(^{2+}\) (instead of Mg\(^{2+}\)) together with a certain conformation of bound nucleotide, competition between Mg\(^{2+}\) and Ca\(^{2+}\) for binding to nucleotide in solution will make that conformation of bound nucleotide unfavourable; thus, the high Ca\(^{2+}\) concentration will not result in Ca\(^{2+}\) binding, but only in destabilization of a
previously bound Mg$^{2+}$ (together with
destabilization of that particular conformation of
nucleotide), as in fact found in the E1.AMPPCP
crystals, which have only one Mg$^{2+}$ ion bound,
instead of two in the E1.ADP.AlFx or
E2.MgF$_4$.ADP crystals (4-6) (see Supplemental
Material for additional discussion).

Having said that the E1.ADP.AlFx and
E1.AMPPCP forms in solution are made more
similar to each other by the presence of a high
Ca$^{2+}$ concentration (and detergent), we can,
conversely, discuss the fact that under usual
conditions in the test tube they seem to be
different. To start with a preliminary point, we
can exclude the possibility that this is due to the
reversible nature of AMPPCP binding, as
opposed to the almost irreversible nature of
ADP.fluoroaluminate binding to ATPase: if the
exit pathway for Ca$^{2+}$ were to be occluded when
AMPPCP sits at its binding site, but open when
AMPPCP has dissociated, the rate of Ca$^{2+}$
dissociation should be slowed down to zero
at high [AMPPCP], in proportion with the
average statistical occupation by AMPPCP of
this site, even in the case of extremely fast
dissociation and rebinding of AMPPCP. This is
not the case, as AMPPCP concentrations much
higher than its $K_d$ (e.g. 100 fold higher, i.e.
leaving unoccupied only 1 % of the sites) only
have a moderate effect on the rate of Ca$^{2+}$
dissociation (Figure 1B).

A more subtle possibility has been
suggested, namely that even with nucleotide
bound, the ATPase.AMPPCP complex (and
especially the critical M1-M2 hairpin)
experiences fluctuations that are sufficient to
allow Ca$^{2+}$ dissociation, whereas this flexibility
might be lost upon covalent phosphorylation or
formation of the quasi-covalent transition state
complex (4). Although we agree with the
existence of such flexibility in the
ATPase.AMPPCP complex and with its possible
reduction in the E1.ADP.AlFx transition state
complex (especially as concerns slow and large-
amplitude fluctuations), we however think that
this putative slowing down of the protein
dynamics must also be accompanied by a
significant shift of the average conformation
of the protein, because invoking the protein
dynamics alone cannot account for the
differences in Trp fluorescence experimentally
observed for the E1.AMPPCP and E1.ADP.AlFx
forms (e.g. Figs 6 & 8). This is because this
putative reduction in flexibility due to formation
of a transition state analog at the catalytic site is
likely not to affect much, in residues located at
some distance from the catalytic site, those
movements that are fastest and of smallest
amplitude: since the Trp residues in the
transmembrane ATPase segments are located far
from the catalytic site, this putative reduction in
flexibility can therefore be expected not to affect
much the fast movements (if any) contributing to
sub-nanosecond or nanosecond relaxation of the
excited state of Trp residues, susceptible to bias
the statistical average by Trp fluorescence of
conformational states. Experimentally, fast and
small amplitude movements of residues in the
ATPase transmembrane domain indeed do not
seem to be reduced in crystallized ATPase
transition state complexes, compared with more
usual ATPase complexes, as judged from
B factors in the corresponding crystals (4-7); in
fact, the resolution was reportedly even slightly
better with E1-AMPPCP (4; C. Toyoshima,
unpublished result). Thus, the finding that the
ATPase Trp fluorescence does change when
going from E1.AMPPCP to E1.ADP.AlFx
implies that the dynamic equilibrium between
forms of high and low fluorescence is poised
differently in the two complexes (and not only
slowed down). In other words, even if limited
flexibility is present and reduces the frequency
with which the E1.ADP.AlFx species may
explore the entire conformational space, this
species has been stabilized by ADP.AlFx in an
average state different from the average state
experienced by the E1.AMPPCP complex. Of
course, whether the ATPase complex with
ADP.AlFx corresponds to an extreme form of
the complex with AMPPCP (not representative
of the average form of the latter) or to a different
one, is then a matter of different phrasing for the
same reality.

How the average conformation of the
E1.AMPPCP complex looks like in solution, we
do not know yet. This conformation might differ
in the architecture of the polypeptide chain
(hence the changes in Trp fluorescence), but also
in the conformation of the bound nucleotide. A
priori, it is clear that ATPase phosphorylation
from ATP requires that the $\gamma$-phosphate of bound
nucleotide comes close to the phosphorylatable
Asp$^{351}$ residue, i.e. requires that ATP, bound in
particular to domain N of the ATPase, adopts an
extended conformation (as found in the
crystalline structures) allowing its phosphate
chain to reach domain P, where Asp\textsuperscript{351} resides. It is also becoming clear from available structures that this extended conformation of nucleotide will act as a cross-linker between the N- and P-domains (3-5, 9), stabilizing thermal movements of the polypeptide chain and therefore contributing to occlusion of the Ca\textsuperscript{2+} ions bound at the transport sites, especially if the (kinked) M1 helix blocks flickering of residue Glu\textsuperscript{309}, gating the exit from the Ca\textsuperscript{2+} binding pocket. At this point, though, it might be of interest to recall that different lines of evidence have already suggested that binding of nucleotide to Ca\textsuperscript{2+}-saturated ATPase might result (at least in the case of ATP) in the formation of more than one form of ATPase.nucleotide complex (13, 37-46, 18; see further comments in Supplemental Material).

Going further along this line, it has even been suggested that conformations of the ATPase.nucleotide complex still allowing Ca\textsuperscript{2+} dissociation could be conformations where the bound nucleotide has adopted a \textit{folded} conformation (30), for instance with the phosphate chain close to Thr\textsuperscript{441} or Glu\textsuperscript{439} in the N-domain as suggested by experiments in the presence of Fe\textsuperscript{2+}, thought to replace Mg\textsuperscript{2+} (47, 48). Such a bent conformation has already long ago been inferred from NMR experiments in the presence of Mn\textsuperscript{2+}, for Co(NH\textsubscript{3})\textsubscript{4}ATP bound to Na\textsuperscript{+},K\textsuperscript{+}-ATPase (49). The conformation of a given nucleotide in solution, say AMPPCP, is highly dynamic (with average conformation depending on conditions and not necessarily poised towards an extended conformation), and ATPase-nucleotide complexes could indeed exist in two (or more) forms, with bound nucleotide either in a bent conformation providing little stabilization of the cytoplasmic domains and therefore not preventing Ca\textsuperscript{2+} exchange, or in the extended conformation found in the crystals, a conformation providing stabilization of the N-P interaction and therefore favourable for Ca\textsuperscript{2+} occlusion and phosphorylation.

Our results fit best with this line of thoughts, and might imply that in solution under usual Ca\textsuperscript{2+} conditions, most of the ATPase-nucleotide complexes might \textit{not} adopt the extended conformation seen in the crystal, but might have their nucleotide bound in a different conformation, for instance the conformation suggested by Stewart et al. for Na\textsuperscript{+},K\textsuperscript{+}-ATPase, and simultaneously would have Ca\textsuperscript{2+} sites with an \textit{unblocked} exit towards the cytosol (accounting for the fairly fast dissociation of Ca\textsuperscript{2+} observed in \textsuperscript{45}Ca\textsuperscript{2+} filtration or quin2 experiments). The fraction of complexes with the same extended conformation as that found in the crystal would only get larger in the presence of high Ca\textsuperscript{2+} (and detergent). Note that even under those conditions, this fraction does not necessarily have to reach 100 %, as crystallization constraints are known to have the potential to \textit{select} particular protein conformations from many possible ones in solution: this is especially fascinating in crystals where the unit cell is found to contain monomers in two different conformations (e.g. ref. 50); another example is provided by the fact that 3D-crystals of the E1.Ca\textsubscript{2} form of Ca\textsuperscript{2+}-ATPase show a significantly more open cytosolic domain than previous 2D-crystals of the same form (1, 51).

Back to our main conclusion that the E1.AMPPCP average structure under usual conditions in solution probably differs from that illustrated in the crystal, the fact that a crystalline structure does not necessarily reflect the average conformation of a protein in solution is well known to crystallographers. But for more naive biochemists, the \textit{caveat} derived from the present results will have to be kept in mind for interpreting present and future structures in terms of average conformational changes of the ATPase during its catalytic cycle. Fortunately enough, in the case of the already published E1.AMPPCP form, the (rare?) conformation of the complex selected in the crystalline form actually turns out to be a very interesting one, in fact the \textit{most desirable} one, as it reveals the ATPase transient conformation in which the nucleotide \textgamma-phosphate approaches Asp\textsuperscript{351}, leading to phosphorylation at the catalytic site and occlusion at the Ca\textsuperscript{2+} transport sites.

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**FOOTNOTES**

**Abbreviations** : SR, sarcoplasmic reticulum; ATPase, adenosine triphosphatase; AMPPCP, adenosine 5’-(β,γ-methylene)triphosphate; quin2, 2-[(2-amino-5-methylphenoxy)methyl]-6-methoxy-8-aminoquinoline-N,N,N’,N'-tetraacetic acid; EDTA, ethylenediamine-tetraacetic acid; EGTA, (ethylenbis-(oxethylenenitrilo)]tetraacetic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DTNB, Ellman’s reagent, 5,5'-dithio-bis(2-nitrobenzoic acid); PK, proteinase K; TCA, trichloroacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; C12E8, octaethylene glycol monododecyl ether; DDM, dodecylmaltoside; DPC, dodecylphosphocholine.
FIGURE LEGENDS

Figure 1. AMPPCP stimulates or slightly reduces the rate of quin2-induced Ca$^{2+}$ dissociation from Ca$^{2+}$-ATPase, depending on Mg$^{2+}$; this modulation by Mg$^{2+}$ differs from that for other nucleotides. The effect of AMPPCP (Panels A & B) or other nucleotides (Panel C) on the rate of Ca$^{2+}$ dissociation from SR Ca$^{2+}$-ATPase was measured at various concentrations of Mg$^{2+}$. The buffer also contained 50 mM Mes-Tris (pH 6 and 20°C). Panels A & B, sarcoplasmic reticulum membranes (SR, 0.4 mg/ml) were pre-incubated with calcium (100 µM) in the absence or presence (as indicated on each trace) of 250 µM AMPPCP. This suspension was mixed in a one to one volume in a stopped-flow device with quin2 (400 µM), again in the absence or presence of AMPPCP. Quin2 fluorescence (excited at 312 nm), whose rise reflects Ca$^{2+}$ dissociation from the ATPase, has been normalized to its initial fluorescence level immediately after mixing. Monoexponential fits (to $y = y_0 + a(1-e^{bx}) + c.x$) and experimental data points are superimposed. The experiment illustrated in Panel A was performed at 3 mM Mg$^{2+}$, in the absence or presence of 250 µM AMPPCP. Similar measurements were performed at various AMPPCP and Mg$^{2+}$ concentrations (some of them are shown in Figure III of Supplemental Material), and measured rate constants are plotted in Panel B. For Panel C, similar experiments were performed in the absence (circles) or presence of various nucleotides, now added together with quin2 at twice their final concentration after mixing: AMPPCP again (squares) (results were similar to those with the other protocol), ATP$_{γ}$S (diamonds), or ADP (triangles) (final concentrations were 250 µM, 250 µM, and 1 mM, respectively). Note that in a control experiment, 250 µM AMPPCP alone was preincubated with 100 µM Ca$^{2+}$ (in the absence of SR vesicles or Mg$^{2+}$) and then mixed one to one with quin2 : under these conditions, the fluorescence trace remained flat, implying that Ca$^{2+}$ bound to the nucleotide dissociates within the mixing time of the experiment.

Figure 2. Effect of Ca$^{2+}$ and Mg$^{2+}$ on AMPPCP binding to ATPase: Mg$^{2+}$ reduces the ATPase affinity for AMPPCP, whereas a high millimolar Ca$^{2+}$ increases it. SR membranes (here at 0.08 mg/ml) were suspended in a medium containing 50 mM Mes-Tris (pH 6 and 20°C). Panels A & B, EGTA (0.5 mM) was first added, followed for Panel B by 3 mM Mg$^{2+}$ (not shown); then, AMPPCP was sequentially added, at the final total concentrations indicated (in µM). This involved only minimal dilution (typically 2 µl of concentrated ligand into 2 ml), and these small dilution-induced artifacts have been corrected for. Panels C&D, EGTA (0.5 mM) was again added first; this was followed by addition of Ca$^{2+}$ (0.6 mM total concentration, hence 0.1 mM free Ca$^{2+}$) and for Panel D, Mg$^{2+}$; then, AMPPCP was again sequentially added, at the final total concentrations indicated (in µM). Panel F, same as for Panel D but with an extra addition of 10 mM Ca$^{2+}$ (double arrow) before AMPPCP. Here, fluorescence emission at 330 nm was recorded with excitation at 300 nm (respective spectral bandwidths were 10 and 5 nm) in order to minimize inner filter effects due to the highest nucleotide concentrations. The equilibrium dissociation constant for nucleotide estimated from each curve has been indicated on the corresponding Panel; Panel E shows the plots corresponding to experiments performed in the presence of 3 mM Mg$^{2+}$.

Figure 3. AMPPCP binding at millimolar Ca$^{2+}$ (but not submillimolar Ca$^{2+}$) almost fully protects ATPase from proteolysis by proteinase K. SR Ca$^{2+}$-ATPase (2 mg/ml) was treated for various periods with Proteinase K (0.03 mg/ml) at 20°C in proteolysis buffer containing 100 mM Mes-Tris (pH 6.5), 100 mM NaCl, 1 mM Mg$^{2+}$ and either 0.1 mM Ca$^{2+}$ (Top Panel) or 10 mM Ca$^{2+}$ (Bottom Panel), in the absence (lanes 2-5) or presence (lanes 6-9) of 0.5 mM AMPPCP, and the resulting fragments were separated by SDS-PAGE on a Laemmli gel and stained with Comassie Blue. Lanes 1 show molecular mass standards (Pharmacia LMW).

Figure 4. A millimolar Ca$^{2+}$ concentration, together with AMPPCP, slows down reaction of DTNB with Ca$^{2+}$-ATPase SH groups significantly.
SR vesicles (0.15 mg protein/ml, i.e. about 1 µM ATPase) were added to a medium (25 °C) containing 0.5 mM DTNB, 100 mM Tricine-Tris (pH 8), 100 mM NaCl, 1 mM Mg²⁺ and either 0.05 mM Ca²⁺ or 10 mM Ca²⁺, in the absence or presence of 0.5 mM AMPPCP. Reaction with DTNB was monitored by taking full spectra every half a minute, as shown in Panels A & B for the experiments performed at 10 mM Ca²⁺ without (A) or with (B) AMPPCP. After 25-30 minutes, 1 mg/ml SDS was added (resulting in SR vesicle solubilization), to allow more slowly-reacting SH groups to nevertheless react with DTNB, and lastly L-cysteine was added (20 µM), for internal calibration. In Panels C and D, we plotted optical densities at 430 nm and 550 nm, respectively, for all four experiments; the former wavelength is intended to reveal SH reaction with DTNB, while the latter wavelength will reveal simple turbidity changes (which remain minimal under these conditions). Conditions for the various traces in Panels C & D are: 10 mM Ca²⁺ + AMPPCP (continuous lines); 10 mM Ca²⁺ only (dashed lines); 0.05 mM Ca²⁺ + AMPPCP (dash-dot-dot lines); 0.05 mM Ca²⁺ only (dotted lines). On the Y scale on the right of Panel C, the tick spacing (ΔOD₄₃₀=0.24) corresponds to reaction with DTNB of about 20 SH groups per ATPase, the number of ATPase groups expected to be reactive to DTNB. In Panel C, the horizontal dashed line represents the OD₄₃₀ (about 0.08) that should be observed, because of turbidity alone, immediately after SR vesicles addition: this dashed line makes it clear that in all cases 1-2 SH groups react with DTNB very rapidly.

Figure 5. The simultaneous presence of AMPPCP and a high millimolar ⁴⁰Ca²⁺ concentration does slow down ⁴⁵Ca²⁺-⁴⁰Ca²⁺ exchange considerably, to a larger extent than the same concentration of Mg²⁺ together with only 1 mM ⁴⁰Ca²⁺.

For these measurements, 1 ml of ATPase-containing SR membrane suspension (0.25 mg SR protein/ml) was loaded onto a Millipore nitrocellulose (HA) filter (0.45 µm pore diameter). The buffer consisted 50 mM Mes-Tris at pH 6 and 20°C, supplemented with 3 mM Mg²⁺. The SR membranes had been previously equilibrated for 5-15 minutes with 50 µM (Panel A) or 25 µM (Panel B) ⁴⁵Ca²⁺ (plus 50 µM [³H]glucose as a volume marker). Panel A, the adsorbed membranes were perfused for various periods of time with Mg²⁺-containing buffer supplemented with 10 mM EGTA (open circles), 1 mM ⁴⁰Ca²⁺ (open triangles), 10 mM ⁴⁰Ca²⁺ (open squares), 1 mM ⁴⁰Ca²⁺ and 0.25 mM AMPPCP (closed triangles), or 10 mM ⁴⁰Ca²⁺ and 0.25 mM AMPPCP (closed squares). Panel B, the adsorbed membranes were then perfused for various periods of time with Mg²⁺-free buffer supplemented with either 10 mM EGTA (open circles), 10 mM EGTA plus 0.5 mM AMPPCP (closed circles), 10 mM ⁴⁰Ca²⁺ (open squares), 10 mM ⁴⁰Ca²⁺ plus 0.5 mM AMPPCP (closed squares), or 1 mM ⁴⁰Ca²⁺ plus 10 mM Mg²⁺ and 0.5 mM AMPPCP (closed upside down triangles). Data points are the mean of triplicate experiments. The slightly lower initial binding in Panel B compared to Panel A is due to the fact that at pH 6 in the presence of 3 mM Mg²⁺, the ATPase affinity for ⁴⁵Ca²⁺ is such that 25 µM ⁴⁵Ca²⁺ is not fully saturating. The actual ⁴⁵Ca²⁺ binding levels at time zero, i.e. before any perfusion, were higher by 1-2 nmol/mg (not shown) than those shown here after the shortest perfusion period (0.03 s), because ⁴⁵Ca²⁺ also binds to non-specific sites (e.g. negatively charged lipids) with very fast dissociation rates. The residual amount of ⁴⁵Ca²⁺ after 4 s perfusion is due to ⁴⁵Ca²⁺ having slowly equilibrated passively in the lumen of the vesicles, as previously discussed (12).

Figure 6. In the presence of 50 µM Ca²⁺, ADP/AlF₄ raises the ATPase intrinsic fluorescence to a level higher than that of E₁.AMPPCP, but the difference is less prominent in the presence of 10 mM Ca²⁺. SR vesicles (0.1 mg/ml) were suspended in a medium containing 100 mM KCl, 50 mM Mops-Tris and 5 mM Mg²⁺ at pH 7 and 20°C, and intrinsic fluorescence was examined with excitation and emission wavelengths of 295 and 330 nm, respectively (respective spectral bandwidths were 2 and 10 nm). Initial addition to the cuvette of EGTA first (240 µM, to chelate contaminating and/or endogenous Ca²⁺, a few micromolar total) and then excess Ca²⁺ (285 µM, to reach a free [Ca²⁺] of about 50 µM and thus obtain the “Ca⁺⁺E₁” form) served as an “internal calibration” of the fluorescence changes. Then, various ligands were added: either ADP (100 µM), KF (1 mM), AlCl₃ (50 µM) and then EGTA again (2 mM) (Panel A), or AMPPCP (100 µM) followed by another identical addition of AMPPCP to check for saturation (Panel B). Panels C & D, the Ca²⁺ concentration in the cuvette was...
first brought up to 10 mM (double arrow), before addition of the same ligands as in Panels A & B, respectively. The small dilution-induced artefacts have been corrected for.

Figure 7. Trp residues in E1 (blue) and E1.AlFx.ADP (red), superimposed with the two ribbon structures (blue and red, respectively).

The ribbon structure of E1 (1SU4) is colored bluish, and that of E1.AlFx.ADP (1WPE) is colored reddish. The different Trp residues are indicated (blue (E1), red (E1·AlFx·ADP) or violet (both at the same positions) labels), located at positions 50 (top of M1 or M1’), 77 (bottom of M1), 107 (top of M2), 272 (bottom of M3), 288 (bottom of M4), 794 (M6), 832 (top of M7), 854 and 855 (bottom of M7), 928 and 932 (top of M9) and 967 (bottom of M10). 1WPE and 1SU4 have been superimposed by fitting the transmembrane domains. This Figure emphasizes that, as a result of the upwards movement of M1 and the bending of the top amphipathic part (M1’) of M1 when going from 1SU4 to 1WPE, Trp77 at the bottom of M1 moves upwards, leaving the membrane border and entering the hydrophobic region of the membrane; Trp77 therefore is a likely candidate for being responsible for a higher fluorescence; Trp107 at the top of M2 also slightly shifts upwards (thus possibly going from the membrane border to a more hydrophilic environment), while Trp50 in M1 (previously buried within the transmembrane helices) has a much more exposed position (presumably at the phospholipid interface), but in these two cases rearrangement of lipids might cancel in part the expected drop in fluorescence. The other Trp residues in the membrane only change position to a much lower extent. This figure was prepared with TurboFRODO.

Figure 8. In the presence of a solubilizing concentration of detergent (here DDM at 2.5 mg/ml), the effect of 10 mM Ca\(^{2+}\) on ADP.AlFx- and AMPPCP-induced changes is even more prominent.

Experiment similar to that in Figure 6, except that 2.5 mg/ml dodecylmaltoside (DDM) was added to the cuvette after the initial calibration of the Trp fluorescence signal with EGTA and Ca\(^{2+}\). SR vesicles (0.1 mg/ml) were suspended in a medium containing 100 mM KCl, 50 mM Mops-Tris and 5 mM Mg\(^{2+}\) at pH 7 and 20°C, and intrinsic fluorescence was examined with excitation and emission wavelengths of 295 and 330 nm, respectively (respective spectral bandwidths were 2 and 10 nm). Initial addition to the cuvette of EGTA first (240 µM, to chelate contaminating and/or endogenous Ca\(^{2+}\), a few micromolar total) and then excess Ca\(^{2+}\) (285 µM, to reach a free [Ca\(^{2+}\)] of about 50 µM and thus obtain the “Ca\(^{2+}\)E1” form) served as an “internal calibration” of the fluorescence changes. Then, various ligands were added: either DDM (2.5 mg/ml), ADP (100 µM), KF (1 mM) and AlCl\(_3\) (50 µM) (Panel A), or DDM (2.5 mg/ml) and AMPPCP (100 µM) followed by another identical addition of AMPPCP to check for saturation (and finally a high Ca\(^{2+}\) (10 mM, double arrow)) (Panel B). Panels C & D, the Ca\(^{2+}\) concentration in the cuvette was first brought up to 10 mM (double arrow), before addition of the same ligands as in Panels A & B, respectively. The small dilution-induced artefacts have been corrected for.
Figure 1. AMPPCP stimulates or slightly reduces the rate of Ca\(^{2+}\) dissociation from Ca\(^{2+}\)-ATPase, depending on Mg\(^{2+}\); this modulation by Mg\(^{2+}\) differs from that for other nucleotides.
Figure 2. Effect of Ca$^{2+}$ and Mg$^{2+}$ on AMPPCP binding to ATPase: Mg$^{2+}$ reduces the ATPase affinity for AMPPCP, but a high millimolar [Ca$^{2+}$] increases this affinity.
Figure 3. AMPPCP binding at millimolar Ca\(^{2+}\) (not submillimolar Ca\(^{2+}\)) almost fully protects ATPase from proteolysis by proteinase K at pH 6.5 and 1mM Mg\(^{2+}\).

A: \([\text{Ca}^{2+}] = 0.1\) mM

B: \([\text{Ca}^{2+}] = 10\) mM
Figure 4. A millimolar Ca\(^{2+}\) concentration, together with AMPPCP, slows down reaction of DTNB with ATPase SH groups significantly.
Figure 5. The simultaneous presence of AMPPCP and a high [Ca2+] slows down 45Ca2+-40Ca2+ exchange considerably.
Figure 6. ADP/AlFx raises the ATPase intrinsic fluorescence to a level *higher* than that of E1.AMPPCP, but the difference is less prominent in the presence of 10 mM Ca\(^{2+}\) than in the presence of 0.05 mM Ca\(^{2+}\).
Figure 7. Trp residues in E1.2Ca\textsuperscript{2+} (1SU4, violet) and E1. AIF\textsubscript{x} .ADP (1WPE, brown)
Figure 8. In the presence of a solubilizing concentration of detergent (here DDM at 2.5 mg/ml, i.e. about 5mM), the effect of 10 mM Ca\(^{2+}\) on ADP.\textit{AlF}_x^- and AMPPCP-induced changes is even more prominent.
SUPPLEMENTAL MATERIAL

For the Ms by Martin Picard, Chikashi Toyoshima and Philippe Champeil:

The average conformation at micromolar [Ca\(^{2+}\)] of Ca\(^{2+}\)-ATPase with bound nucleotide differs from that adopted with the transition state analog ADP.AIF\(_x\) or with AMPPCP under crystallization conditions at millimolar [Ca\(^{2+}\)].

Possible alternative reasons for the effect of high Ca\(^{2+}\) on the properties of the ATPase.AMPPCP complex.

We considered the possibility that a high Ca\(^{2+}\) concentration could have resulted in some modification of the phospholipid/water interface. In fact, the top part of M1 helix, dubbed M1’, is an amphipathic helix with four negatively charged residues on the same side of the helix (LWE\(_{51}\)LVIE\(_{55}\)QFE\(_{58}\)D\(_{59}\)LLVR\(_{63}\)…), and in E1.AMPPCP and E1.ADP.AIF\(_x\) crystalline forms, M1’ is found to kink and lean on what normally is the membrane interface. It could therefore be imagined that by neutralizing phosphates at this interface, a high Ca\(^{2+}\) concentration favours the kinked conformation. In this case, the extra Ca\(^{2+}\) ions would be bound with poor affinity at this interface, and would therefore probably remain undetected. However, the fact that the effect of 10 mM Ca\(^{2+}\) was even more apparent in solubilized ATPase than in native membraneous ATPase, including in the presence of detergent concentrations likely to delipidate the protein to a large extent, does not favour this explanation. We then considered another explanation for stabilization by Ca\(^{2+}\) of the E1.AMPPCP structure with a kinked M1’: by binding (again with poor affinity, hence probably in an undetectable way in X-ray diffraction patterns) to acidic residues in M1’, and especially to Asp\(_{59}\), Ca\(^{2+}\) could destabilize the salt bridge between Asp\(_{59}\) and Arg\(_{63}\) involved in formation of the straight M1 helix (1SU4). In this view, the positive influence of detergent on the effect of high Ca\(^{2+}\) could be due to the fact that the transmembrane architecture found in the E1.ADP.AIF\(_x\) form might occupy a larger cross section in the membrane than that for E1.Ca\(_{2+}\), and therefore would be favoured after the lateral pressure exerted by phospholipids is relieved by solubilization.

Assuming that extra Ca\(^{2+}\) is not bound in the E1.AMPPCP crystals, the reason for the particular effect of high Ca\(^{2+}\) and Mg\(^{2+}\) can also be discussed in the context of the hypothesis that bound nucleotide may adopt different conformations on the ATPase, for example either bent or extended conformations. For instance, a priori, as the presence of Mg\(^{2+}\) between Asp\(_{351}\) and Asp\(_{703}\) would reduce electrostatic repulsion between the nucleotide phosphate chain and Asp\(_{351}\), it is certainly understandable that Mg\(^{2+}\) could favour an extended (cross-linking) conformation of bound nucleotide, so that Ca\(^{2+}\) dissociation from its sites in the presence of the Mg\(^{2+}\).AMPPCP complex would be slowed down compared to dissociation in the presence of Mg\(^{2+}\)-free AMPPCP (Figures 1B & 5B). But this electrostatic effect, alone, does not allow to understand the different dependence on Mg\(^{2+}\) found for various nucleotides (e.g. ATP-\(\gamma\)S, for which the presence of Mg\(^{2+}\) accelerates Ca\(^{2+}\) dissociation instead of slowing it down, see Figure 1C); it is not obvious either with this sole electrostatic effect to understand why a high millimolar Ca\(^{2+}\), instead of Mg\(^{2+}\), could further slow down Ca\(^{2+}\) dissociation in the presence of AMPPCP, and why Mg\(^{2+}\) and Ca\(^{2+}\) would have different effects on the conformation of the...
complex. Moreover, if we accept the idea that under usual conditions the major form of bound nucleotide is a folded one, as part of the evidence for this folded form was derived from experiments where Fe\(^{2+}\) was supposed to sit at a Mg\(^{2+}\)-binding site we must simultaneously admit that this form exists in the presence of Mg\(^{2+}\), not only in its absence. We are therefore led to accept that under usual conditions the ATPase.nucleotide complex with the folded nucleotide probably contains two Mg\(^{2+}\) ions instead of one (there are indications for this in the literature, e.g. in ref. 27), for instance a first one interacting with the folded phosphate chain (and in some cases close to Thr\(^{341}\) as suggested by the Fe\(^{2+}\) experiments) and a second one close to Asp\(^{351}\) and Asp\(^{703}\) of the P-domain; two Mg\(^{2+}\) ions are in fact found together with bound ADP in the crystalline E1.ADP.AIFxF complex (4, 6) or the crystalline E2.ADP.MgF\(_4\) complexes (6). In this context, if we assume that the Mg\(^{2+}\) site close to Glu\(^{339}\) and Thr\(^{341}\) cannot accommodate Ca\(^{2+}\) (instead of Mg\(^{2+}\)) together with bound (folded) AMPPCP (although AMPPCP free in solution of course binds Ca\(^{2+}\)), it is understandable that a high Ca\(^{2+}\) concentration will make the folded conformation of bound AMPPCP unfavourable compared with the extended one, and that this site for Mg\(^{2+}\) will remain metal-free (as found in the E1.AMPPCP crystals). In other words, the high Ca\(^{2+}\) concentration will not result in Ca\(^{2+}\) binding, but only in destabilization of a previously bound Mg\(^{2+}\), together with destabilization of the folded conformation of AMPPCP.

Further comments on previous evidence concerning the existence of different forms of ATPase.nucleotide complexes.

Different lines of evidence have already suggested the possibility that binding of nucleotide to Ca\(^{2+}\)-saturated ATPase may result in the formation of more than one form of ATPase, at least in the case of Mg.ATP. This was originally deduced from detailed time-resolved phosphorylation experiments, the conclusion of which was that the rate-limiting step for ATPase phosphorylation was not phosphorylation itself, but was a conformational change of the E1.Ca\(_2\).ATP complex to a similar but "activated" "E1.Ca\(_2\).ATP complex, for which phosphorylation occurred faster than 1000 s\(^{-1}\) (13). The existence of a slowly exchanging form of bound nucleotide had already been suggested from the fact that in some cases, phosphoenzyme increases after dilution of radioactive ATP with unlabelled ATP (Fig 6 in ref. 37; Fig 4 in ref. 38; Fig 3 in ref. 13). The existence of such an activated form of nucleotide.ATPase complex was considered (39) to be a possible reason for the spectral change of bound spin-labelled iodoacetamide noted long ago to occur in the presence of Ca\(^{2+}\) and various nucleotides (40-42), and it might correlate with the second phase of the changes in fluorescence of EDANS-labelled Ca\(^{2+}\)-ATPase after ATP addition (43). If the existence of different major forms of nucleotide complexes with Ca\(^{2+}\)-saturated Ca\(^{2+}\)-ATPase is accepted (of course, many different ATPase forms are rapidly interconverting because of the protein dynamics), it is then fairly standard to suggest that crystallization constraints have probably selected a limited number of these forms).

The idea that some (few, in fact) of the conformations of the non-covalent complexes of ATPase with nucleotides already have their Ca\(^{2+}\) transport sites already blocked would nicely fit with the established fact that the mere binding of Cr.ATP to Ca\(^{2+}\)-ATPase, in the absence of phosphorylation, results in slow occlusion of Ca\(^{2+}\) (44, 45). It is fair to mention that it would apparently contradict the idea, that one of us previously put forward, that occlusion of the Ca\(^{2+}\) transport sites does not immediately accompany ATPase phosphorylation, but rather follows the departure of ADP (46), an apparent contradiction which, however, might reveal the previously noted relative independence (18), again due to protein dynamics, of individual sub-conformations in the transmembrane domain and in the cytosolic domains, respectively.
Figure I. AMPPCP does not block $^{45}$Ca$^{2+}$ dissociation from the ATPase, while preincubation with ADP.AlF$_4$ does occlude Ca$^{2+}$ (as monitored by rapid filtration).

Figure I. Effect of AMPPCP and ADP.AlF$_4$ on the rate of Ca$^{2+}$ dissociation from SR Ca$^{2+}$-ATPase, as deduced from $^{45}$Ca$^{2+}$ filtration measurements.

For these measurements, 1 ml of ATPase-containing SR membrane suspension (0.25 mg SR protein/ml) was loaded onto a Millipore nitrocellulose (HA) filter (0.45 µm pore diameter). The buffer consisted of 100 mM KCl, 5 mM Mg$^{2+}$ and 50 mM Mops-Tris at pH 7 and 20°C; SR membranes had been equilibrated for 5-15 minutes in this buffer, together with 50 µM $^{45}$Ca$^{2+}$ plus 50 µM $[^3]$H]glucose as a volume marker (circles are for control) and, in some cases, 250 µM or 2.5 mM AMPPCP (closed or open triangles), or again 100 µM ADP + 1 mM KF + 50 µM AlCl$_3$ (squares; ADP was in fact added first to the SR batch, and KF and AlCl$_3$ were added subsequently, after a few minutes); this incubation was performed in the absence or presence (closed or open symbols, respectively) of 10 µg/ml of the Ca$^{2+}$ ionophore A23187. The SR membranes adsorbed on the filter were then perfused for various periods of time with buffer supplemented with 1 mM EGTA, again in the absence or presence of AMPPCP or ADP + fluoroaluminate. Data points were measured in triplicates, and averaged.

Comments to Figure I. The amount of $^{45}$Ca$^{2+}$ passively bound to SR vesicles (i.e. at t=0) measured was about 14 nmol/mg, out of which about 12 nmol/mg dissociated rapidly, consistent with an ATPase content of about 6 nmol/mg protein. The residual amount of $^{45}$Ca$^{2+}$ found after 2 s perfusion (closed symbols), which was further reduced when ionophore was included in the preincubation medium (open symbols), is presumably due to $^{45}$Ca$^{2+}$ having slowly equilibrated passively in the lumen of the vesicles, as previously found at pH 6 (and discussed in ref. 12). AMPPCP (triangles) at either 250 µM or 2.5 mM hardly affected the rate of dissociation of $^{45}$Ca$^{2+}$. When SR vesicles were preincubated with ADP and fluoroaluminate in the absence of ionophore and subsequently perfused with EGTA, $^{45}$Ca$^{2+}$ was not released from the vesicles, but the amount of trapped $^{45}$Ca$^{2+}$ was initially higher (closed squares). The amount of trapped $^{45}$Ca$^{2+}$ increased slowly during mere preincubation with ADP (data not shown) and was reversed by ionophore, suggesting that this slow uptake was due to the presence in our vesicles of muscle adenylate kinase, a well known contaminant of SR preparations. In the presence of ionophore during preincubation with ADP and fluoroaluminate (open squares), the initial $^{45}$Ca$^{2+}$ binding level was normal and $^{45}$Ca$^{2+}$ was again not released from the membranes (except for a very small proportion, presumably bound to non-specific sites), indicative of $^{45}$Ca$^{2+}$ true occlusion.
**Figure II. Changes in quin2 fluorescence under different situations involving Ca\(^{2+}\) dissociation from quin2 or Ca\(^{2+}\) binding to it.**

The buffer consisted of 50 mM Mes-Tris at pH 6 and 20°C. Panel A, for the experiment illustrated by the top trace, a solution containing 400 µM quin2 and 500 µM Ca\(^{2+}\) was mixed in a one to one volume, in a stopped-flow device, with a solution containing 20 mM EDTA. For the bottom trace, a solution containing 20 µM EDTA and 20 µM Ca\(^{2+}\) in buffer was mixed with a solution containing 400 µM quin2. For the intermediate trace, a solution containing 20 µM EDTA and 100 µM Ca\(^{2+}\) was mixed with 400 µM quin2. Panel B, same trace as the latter one, but plotted on a different scale. Panel C, a solution containing 0.4 mg/ml SR vesicles and 100 µM Ca\(^{2+}\) (in the same buffer) was mixed with 400 µM quin2. In all cases, quin2 fluorescence, excited at 312 nm, was monitored, and plotted as the photomultiplier output. Experimental data points and monoexponential fits (to $y = y_0 + a(1-e^{-bx}) + c.x$) have been superimposed (at this scale, they are indistinguishable).

**Comments to Figure II.** The top trace in Panel A of this Figure shows that when quin2 is first complexed with Ca\(^{2+}\) and then mixed in a one to one volume with a much larger concentration of a non fluorescent chelator like EDTA (EGTA can also be used, depending on pH and Mg\(^{2+}\), which influence the Kd of the non-fluorescent chelator relative to that of quin2), quin2 fluorescence, excited at 312 nm (a wavelength where our mercury-doped Xenon lamp has a high intensity), drops, because the Ca.quin2 complex dissociates. In contrast, when a preformed complex of non-fluorescent chelator with Ca\(^{2+}\) is mixed with excess quin2 (bottom trace in Panel A), the Ca\(^{2+}\)-chelator complex dissociates and the released Ca\(^{2+}\) now binds to quin2 and raises its fluorescence. If excess free Ca\(^{2+}\) is present together with the initial chelator complex (middle trace in Panel A), it binds to quin2 with no delay on this time scale (i.e. within the mixing time of the stopped-flow device, about 3 ms), and the similar time-course of the observed fluorescence rise therefore reflects the rate of Ca\(^{2+}\) dissociation from the complex. All traces are remarkably monophasic (Panels A & B), and nicely reveal the dissociation rates of the...
Ca\(^{2+}\) complexes with either quin2 or the non-fluorescent chelator. If excess quin2 is now mixed with SR membranes pre-equilibrated with Ca\(^{2+}\) (Panel C), the fluorescence rise as a function of time now monitors Ca\(^{2+}\) dissociation from the SR ATPase Ca\(^{2+}\) binding sites. By comparison with the previous control obtained with 20 µM EDTA in Panel B, the amplitude of the quin2 fluorescence rise in Panel C also provides a fair estimate of the concentration of the binding sites in SR ATPase: about 5 µM in the enzyme syringe (which contains 0.4 mg/ml SR protein), i.e. 12-13 nmol/mg protein, a number in agreement with previous estimates of the \(^{45}\text{Ca}^{2+}\) binding stoichiometry in our membranes (12). The monophasic fluorescence rise is generally followed by a hardly visible (but real) much slower upwards drift, which probably corresponds to slow Ca\(^{2+}\) release from the lumen of the vesicles and which is taken into account to estimate the true rate constant for Ca\(^{2+}\) dissociation.
**Figure III.** Effect of AMPPCP on the rate of Ca$^{2+}$ dissociation from Ca$^{2+}$-ATPase at pH 6, in the absence or presence of 20 mM Mg$^{2+}$.

SR 0.4 mg/mL + Ca$^{2+}$ 100 µM ± AMPPCP quin2 400 µM ± AMPPCP (50 mM Mes-Tris ± Mg$^{2+}$, pH 6, 20°C)

Incidentally, note that in the absence of AMPPCP, raising the Mg$^{2+}$ concentration up to 20 mM Mg$^{2+}$ slightly stimulated the rate of Ca$^{2+}$ dissociation, compared to the absence of Mg$^{2+}$ (see Fig 1C in main text), but this stimulation vanished in the additional presence of 100 mM KCl (data not shown).
Figure IV. Effect of ATP\(\gamma\)S on Ca\(^{2+}\) dissociation from Ca\(^{2+}\)-ATPase at pH 6 in the absence or presence of Mg\(^{2+}\).

Sarcoplasmic reticulum membranes (SR, 0.4 mg/ml) were pre-incubated with calcium (100 \(\mu\)M) at pH 6 and 20°C in 50 mM Mes-Tris, in the absence (Panel A) or presence (Panel B) of 3 mM Mg\(^{2+}\). This suspension was mixed vol:vol in a stopped-flow device with quin2 (400 \(\mu\)M), to which 500 \(\mu\)M ATP\(\gamma\)S had been added if indicated. Quin2 fluorescence, excited at 312 nm, was normalized to its initial level after mixing. Monoexponential fits (to \(y = y_0 + a.(1–e^{-bx}) + c.x\)) and experimental data points have been superimposed. Similar measurements were performed at various ATP\(\gamma\)S concentrations. All rate constants are plotted in Panel C as a function of the final ATP\(\gamma\)S concentration.

Comments to Figure IV. It can be noted that in Panel B, the amplitude of the trace corresponding to Ca\(^{2+}\) dissociation was slightly smaller in the presence of ATP\(\gamma\)S than in its absence. A simple explanation for this is that ATPase thio-phosphorylation probably occurred upon mixing, to a small extent, and resulted in occlusion of a fraction of the initially bound Ca\(^{2+}\) ions, making it no longer available for quin2. This interpretation was tested by making an additional series of experiments, now with ATP itself instead of ATP\(\gamma\)S. In this case too, the amount of Ca\(^{2+}\) released towards the cytosol as a result of quin2 addition is expected to be diminished because of the competition between Ca\(^{2+}\) dissociation and ATP-induced phosphorylation followed by occlusion (and subsequent release towards the vesicle lumen). When 250 \(\mu\)M of Na\(_2\)ATP (instead of ATP\(\gamma\)S) was present together with quin2, the amplitude of the rise in quin2 fluorescence after mixing was indeed strongly diminished (data not shown), by more than 80 and 90% in the presence of 3 and 20 mM Mg\(^{2+}\), respectively, and also by close to half in the nominal absence of Mg\(^{2+}\) (presumably because of contaminant Mg\(^{2+}\), or because of ATPase phosphorylation from Ca.ATP instead of the normal substrate Mg.ATP). Part of the apparently larger increase of the Ca\(^{2+}\) dissociation rate constant in the presence of Mg\(^{2+}\) compared to its absence is probably due to this reduction in amplitude.
Figure V. Effect of Mg\(^{2+}\) on ATP binding to ATPase: as well known, Mg\(^{2+}\) increases the ATPase affinity for ATP in the absence of Ca\(^{2+}\).

Figure V. Effect of Mg\(^{2+}\) on ATP-induced changes in ATPase fluorescence in the absence of Ca\(^{2+}\).

Panel A, sarcoplasmic reticulum membranes were suspended at 0.08 mg/ml in a medium containing 50 mM Mes-Tris (pH 6 and 20°C). EGTA (0.5 mM) was first added, followed by 0.5 mM EDTA for Panel A and 3 mM Mg\(^{2+}\) for Panel B (not shown); then, ATP was sequentially added, at the final total concentrations indicated (in µM). This involved only minimal dilution (typically 2 µl of concentrated ligand into 2 ml); these small dilution-induced artifacts have been corrected for. Fluorescence emission at 330 nm was recorded with excitation set at 300 nm, to minimize inner filter effects due to nucleotide at the highest concentrations. The equilibrium dissociation constant for nucleotide deduced from each curve has been indicated on the corresponding Panel.

Comments to Figure V. The maximal amplitude of the ATP-induced fluorescence rise was smaller in the absence than in the presence of Mg\(^{2+}\) (Panel A versus Panel B), as previously noted (27), and accordingly addition of Mg\(^{2+}\) after ATP in Panel A raised the fluorescence level further (double arrow in Panel A). Note also that in contrast with the above-illustrated Mg\(^{2+}\)-dependence of the effect of ATP, the effect of ADP binding was not dependent on Mg\(^{2+}\) (data not shown), again as previously reported (27).
Figure VI. In the absence of Mg$^{2+}$, too, the apparent $K_d$ for AMPPCP binding to SR ATPase is sensitive to Ca$^{2+}$ not only when Ca$^{2+}$ binds to the high affinity transport sites, but also when Ca$^{2+}$ is increased to a millimolar value.

**Figure VI.** Effect of Ca$^{2+}$ on AMPPCP-induced changes in ATPase Trp fluorescence in the absence of Mg$^{2+}$ (at pH 6.5): increasing the Ca$^{2+}$ concentration beyond the level required for saturation of the transport sites again increases the ATPase affinity for AMPPCP further.

Same experiment as the one illustrated in Figure 2 of main text, except that the medium now contained 100 mM Mops-NaOH at pH 6.5 (20°C) and no Mg$^{2+}$. Sarcoplasmic reticulum membranes (SR, 0.1 mg/ml) were suspended in this medium, then 0.2 mM EGTA was added, followed for Panels B-D by various total concentrations of Ca$^{2+}$ (EGTA and total Ca$^{2+}$ concentrations are indicated in mM), and then AMPPCP at the final total concentrations indicated (in µM). This involved only minimal dilution (typically 2 µl of concentrated ligand into 2 ml), and in this particular figure, these small dilution-induced artifacts have not been corrected for. Fluorescence emission was recorded at 330 nm, with excitation at 300 nm, to minimize inner filter effects due to nucleotide, but the same pattern was found with excitation at 294 nm, where the Ca$^{2+}$-induced signal is larger (not shown). The equilibrium dissociation constants for nucleotide estimated from each curve have been indicated, close to the free Ca$^{2+}$ concentration prevailing during the assay. In the additional presence in the medium of either 100 mM KCl or 5 mM Mg$^{2+}$, affinities for AMPPCP binding were slightly poorer than those shown here but were again sensitive to variations in Ca$^{2+}$ between 20 µM and 2 mM (not shown).

**Comments to Figure VI.** Note that while nucleotides have been known for long to raise the intrinsic fluorescence level of Ca$^{2+}$-ATPase to a level intermediate between those of the Ca$^{2+}$-free and Ca$^{2+}$-bound forms (e.g. ref.s 27, 23), what we show here and in Figure 2 of the main text is that in the presence of Ca$^{2+}$, AMPPCP brings ATPase fluorescence to a level higher than that of the Ca$^{2+}$-bound form (see more on this below).
Figure VII. AMPPCP binding at millimolar Ca$^{2+}$ (not submillimolar Ca$^{2+}$) almost fully protects ATPase from proteolysis by proteinase K at pH 7.2 and 1mM Mg$^{2+}$.

Experiment similar to the one in Figure 3 of main text, now performed at pH 7.2. SR Ca$^{2+}$-ATPase (2 mg/ml) was treated for various periods with Proteinase K (0.03 mg/ml) at 20°C in proteolysis buffer containing 100 mM Mops-NaOH (pH 7.2), 100 mM NaCl, 1 mM Mg$^{2+}$ and either 0.1 mM Ca$^{2+}$ (Top Panel) or 10 mM Ca$^{2+}$ (Bottom Panel), in the absence (lanes 2-5) or presence (lanes 6-9) of, here, 0.2 mM AMPPCP. Lane 1 shows molecular mass standards (Pharmacia LMW).
Figure VIII. At pH 7.5 instead of pH 8, 10 mM Ca\(^{2+}\) makes SR vesicles aggregation after reaction with DTNB dramatic, but AMPPCP again reduces SH reactivity and aggregation is also reduced.

SR vesicles (0.15 mg protein/ml, i.e. about 1 µM ATPase) were added to a medium (25 °C) now containing 2 mM DTNB, 100 mM Tes-Tris (pH 7.5), 100 mM NaCl, 1 mM Mg\(^{2+}\), and either 0.05 mM Ca\(^{2+}\) or 10 mM Ca\(^{2+}\), in the absence or presence of 0.5 mM AMPPCP. Reaction with DTNB was monitored by taking full spectra every half a minute, as shown in Panels A & B for the experiments performed at 10 mM Ca\(^{2+}\) without (A) or with (B) AMPPCP. After some time, 1 mg/ml SDS was added (resulting in SR vesicle solubilization), to allow more slowly-reacting SH groups to nevertheless react with DTNB, and lastly L-cysteine was added (20 µM), for internal calibration. In Panels C and D, we plotted optical densities at 430 nm (Panel C) and 550 nm (Panel D), respectively. As for Figure 5 in main text, conditions for the various traces in Panels C & D are: 10 mM Ca\(^{2+}\) + AMPPCP (continuous lines); 10 mM Ca\(^{2+}\) only (dashed lines); 0.05 mM Ca\(^{2+}\) + AMPPCP (dash-dot-dot lines); 0.05 mM Ca\(^{2+}\) only (dotted lines). On the Y scale on the right of Panel C, the tick spacing (ΔOD\(_{430}\)=0.24) corresponds to reaction with DTNB of about 20 SH groups per ATPase, the numbers of ATPase groups expected to be reactive to DTNB. In Panel C, the horizontal dashed line represents the OD\(_{430}\) (about 0.08) that should be observed, because of turbidity alone, immediately after SR vesicles addition; the difference between this line and the initial OD\(_{430}\) values reveals the fast-reacting SH groups.
Figure IX. Upon formation of E1Ca₂⁺/ADP/AlF₄⁻ complex, SH reactivity towards Ellman’s reagent is reduced, but not to zero.

Here, the blank spectrum was recorded with DTNB-free buffer. SR vesicles (0.15 mg protein/ml, i.e. about 1 µM ATPase) were first added to a medium (25 °C) containing 100 mM Tes-Tris (pH 7.5), 100 mM NaCl, 1 mM Mg²⁺ and 0.1 mM Ca²⁺, resulting in a simple turbidity spectrum. Panels A & B, 0.5 mM DTNB was then added at time zero. On Panel A, DTNB absorption at short wavelengths is visible, as well as the color development due to DTNB reaction with SH groups, in the quasi-absence of any change in turbidity; on Panel B, OD₄₃₀ and OD₅₅₀ are plotted (continuous and dotted line, respectively). Panels C & D, after SR, 100 µM ADP, 1 mM KF were added, and then 50 µM AlCl₃; unexpectedly, aluminium addition triggered some increase in turbidity. At time zero, DTNB was then added, later followed by SDS and cysteine as in Figure VIII. Panel C shows the recorded spectra, and OD₄₃₀ and OD₅₅₀ are replotted in Panel D (continuous and dotted line, respectively). On the Y scale on the right of Panel C, the tick spacing (ΔOD₄₃₀=0.24) corresponds to reaction with DTNB of about 20 SH groups per ATPase, the numbers of ATPase groups expected to be reactive to DTNB. In Panels B & D, the horizontal dashed lines represent the OD₄₃₀ that should be observed because of turbidity plus DTNB absorption before any reaction of SH groups, again revealing the existence of one or two fast reacting SH groups per ATPase.
Figure X. At pH 6 and 3 mM Mg\textsuperscript{2+}, the presence of 20 mM \textsuperscript{40}Ca\textsuperscript{2+} together with AMPPCP slows down \textsuperscript{45}Ca\textsuperscript{2+}-\textsuperscript{40}Ca\textsuperscript{2+} exchange to a very large extent.

1 ml of ATPase-containing SR membrane suspension (0.25 mg SR protein/ml) was loaded onto a Millipore nitrocellulose (HA) filter (0.45 \(\mu\)m pore diameter). The buffer consisted of 3 mM Mg\textsuperscript{2+} and 50 mM Mes-Tris at pH 6 and 20°C. The SR membranes had been previously equilibrated for 5-15 minutes with 50 \(\mu\)M \textsuperscript{45}Ca\textsuperscript{2+} (plus 50 \(\mu\)M \(\text{[3H]}\)glucose as a volume marker). The adsorbed membranes were then perfused for various periods of time with Mg\textsuperscript{2+}-containing buffer supplemented with 10 mM EGTA (open circles), 1 mM \textsuperscript{40}Ca\textsuperscript{2+} (open triangles), 20 mM \textsuperscript{40}Ca\textsuperscript{2+} (open squares), or 20 mM \textsuperscript{40}Ca\textsuperscript{2+} plus 0.25 mM AMPPCP (closed squares).
**Figure XI.** Poor effect of AlF₄⁻ in the absence of Ca²⁺ on ATPase intrinsic fluorescence, as previously described by Troullier et al. (1992) (ref. 11).

SR vesicles (0.1 mg/ml) were suspended in a medium containing 100 mM KCl, 50 mM Mops-Tris and 5 mM Mg²⁺ at pH 7 and 20°C, and intrinsic fluorescence was examined with excitation and emission wavelengths of 295 and 315 nm, respectively (respective spectral bandwidths were 2 and 10 nm). Additions were: first EGTA (240 µM, to chelate contaminating and/or endogenous Ca²⁺, a few micromolar total), and then KF (1 mM), AlCl₃ (50 µM) and lastly excess Ca²⁺ (285 µM, to reach a free [Ca²⁺] of about 50 µM): the reduced rise induced by the latter addition after a few minutes reveals ongoing inhibition by fluoroaluminate and is consistent with Fig 1 in Troullier et al.
Figure XII. ADP/AlF$_4$ raises the Ca$^{2+}$-ATPase intrinsic fluorescence to a level higher than that of E1.AMPPCP, and in contrast with the rise induced by AMPPCP, this involves Trp residues sensitive to quenching by A23187.

SR vesicles (0.1 mg/ml) were suspended in a medium containing 100 mM KCl, 50 mM Mops-Tris and 5 mM Mg$^{2+}$ at pH 7 and 20°C, and intrinsic fluorescence was examined, here with excitation and emission wavelengths of 295 and 315 nm, respectively (respective spectral bandwidths were 2 and 10 nm). For Panels C & D, 4 µg/ml A23187 was also added to the SR vesicles from the start (hence the initial drop visible on the Figure). Initial addition to the cuvette of EGTA first (240 µM, to chelate contaminating and/or endogenous Ca$^{2+}$, a few micromolar total) and then excess Ca$^{2+}$ (285 µM, to reach a free [Ca$^{2+}$] of about 50 µM and thus obtain the “Ca$_2$E1” form) served as an “internal calibration” of the fluorescence changes. Then, various ligands were added: either ADP (100 µM), KF (1 mM), AlCl$_3$ (50 µM) and then EGTA again (2 mM) (Panels A & C), or AMPPCP (100 µM) followed by another identical addition of AMPPCP to check for saturation (Panels B & D).

Comments: 315 nm was chosen here as the emission wavelength, but formation of the E1.AIFx.ADP form was detectable by Trp fluorescence irrespective of the emission wavelength between 315 nm and 355 nm (data not shown). Similar results were obtained using 5 µg/ml BrA23187 instead of 4 µg/ml A23187 to quench ATPase fluorescence (data not shown). Fluorescence changes (and Ca$^{2+}$ occlusion, as judged from the lack of effect of subsequent addition of EGTA) upon fluoroaluminate addition were also observed in the presence of 0.25 mg/ml of the nonionic detergent C$_{12}$E$_8$ (as well as of Ca$^{2+}$ and ADP), i.e. under solubilizing conditions (e.g. in Figure XIV below).
Figure XIII. In the presence of a solubilizing concentration of detergent (here C₁₂E₈ at 2.5 mg/ml, i.e. about 5mM), the effect of 10 mM Ca²⁺ on ADP.AiFₓ- and AMPPCP-induced changes is prominent, as with DDM.

Experiment similar to that in Figure 8, except that 2.5 mg/ml C₁₂E₈ instead of DDM was added to the cuvette after the initial calibration of the Trp fluorescence signal with EGTA and Ca²⁺. SR vesicles (0.1 mg/ml) were suspended in a medium containing 100 mM KCl, 50 mM Mops-Tris and 5 mM Mg²⁺ at pH 7 and 20°C, and intrinsic fluorescence was examined with excitation and emission wavelengths of 295 and 330 nm, respectively (respective spectral bandwidths were 2 and 10 nm). Initial addition to the cuvette of EGTA first (240 μM, to chelate contaminating and/or endogenous Ca²⁺, a few micromolar total) and then excess Ca²⁺ (285 μM, to reach a free [Ca²⁺] of about 50 μM and thus obtain the “Ca₂E₁” form) served as an “internal calibration” of the fluorescence changes. Then, various ligands were added: either C₁₂E₈ (2.5 mg/ml), ADP (100 μM), KF (1 mM) and AlCl₃ (50 μM) (Panel A), or C₁₂E₈ (2.5 mg/ml) and AMPPCP (100 μM) followed by another identical addition of AMPPCP to check for saturation (and finally a high Ca²⁺ (10 mM, double arrow)) (Panel B). Panels C & D, the Ca²⁺ concentration in the cuvette was first brought up to 10 mM (double arrow), before addition of the same ligands as in Panels A & B, respectively. The small dilution-induced artefacts have been corrected for.
Figure XIV. In the presence of detergent and A23187 used as quencher, the AMPPCP-dependent increase in residual fluorescence is not larger at 10 mM Ca\(^{2+}\) than at 50 µM Ca\(^{2+}\).

Experiment similar to the one illustrated in Figure XII B&D, except for a few modifications, as follows. SR vesicles were suspended in a medium containing 100 mM KCl, 50 mM Mops-Tris and 5 mM Mg\(^{2+}\) at pH 7 and 20°C, and intrinsic fluorescence was examined, here with excitation and emission wavelengths of 295 and 330 nm, respectively (respective spectral bandwidths were 2 and 10 nm). Initial addition to the cuvette of EGTA first (240 µM, to chelate contaminating and/or endogenous Ca\(^{2+}\), a few micromolar total) and then excess Ca\(^{2+}\) (285 µM, to reach a free [Ca\(^{2+}\)] of about 50 µM and thus obtain the “Ca\(^{2+}\)E1” form) served as an “internal calibration” of the fluorescence changes. For Panels B & D, 10 mM Ca\(^{2+}\) was added (double arrow in Panel B). Then, in all cases, C\(_{12}\)E\(_8\) at 0.25 mg/ml was added. Then for Panels C & D, A23187 was also added (7 or 6 µg/ml, respectively), hence the initial drop visible on these Panels. Then, in all cases, AMPPCP was added (100 µM), followed by another identical addition of AMPPCP to check for saturation. A high Ca\(^{2+}\) (10 mM, double arrow) was added at the end for Panels A & C.
Figure XV. The effect of an additional 20 mM Mg\(^{2+}\) on ADP.AlFx- and AMPPCP-induced changes is not similar to that of 10 mM Ca\(^{2+}\).

Experiment similar to that in Figure XIII, except that 20 mM Mg\(^{2+}\) was added (resulting in a final total concentration of 25 mM Mg\(^{2+}\)) before C\(_{12}E_8\). Comparison of Panels B in Figures XIII and XV shows that the additional Mg\(^{2+}\) reduces the AMPPCP-induced fluorescence rise, in contrast with what 10 mM Ca\(^{2+}\) does. Ca\(^{2+}\) was nevertheless able to at least partially revert the effect of Mg\(^{2+}\) (compare Panels B & D in the present Figure XV).
Figure XVI. Using detergent-solubilized ATPase, a millimolar Ca\(^{2+}\) concentration together with AMPPCP again clearly slows down reaction of SH groups with DTNB.

Same experiment as the one illustrated in Figure 4, except that the medium, in addition, contained 2.5 mg/ml C\(_{12}E_8\). Conditions for the various traces in Panel C are: 10 mM Ca\(^{2+}\) + AMPPCP (continuous line); 10 mM Ca\(^{2+}\) only (dashed line); 0.05 mM Ca\(^{2+}\) + AMPPCP (dash-dot-dot line); 0.05 mM Ca\(^{2+}\) only (dotted line). Reaction of DTNB with the SH groups of solubilized ATPase was faster than for membraneous ATPase in Figure 4 and the kinetics of the rise in absorbance was slightly S-shaped, reminiscent of what was previously described in Andersen & Møller (1977), Biochim. Biophys. Acta 485, 188-202.
The average conformation at micromolar [Ca2+] of Ca2+-ATPase with bound nucleotide differs from that adopted with the transition state analog ADP.AIFx or with AMPPCP under crystallization conditions at millimolar [Ca2+].

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